Carcinogenesis vol.21 no.4 pp.653–661, 2000

Increase in mutation frequency in lung of Big Blue® rat by exposure to diesel exhaust

Hiromi Sato[1,4], Hideko Sone[2], Masaru Sagai[3], Kazuo T.Suzuki[4] and Yasunobu Aoki[1,5]

[1]Environmental Health Sciences Division, [2]Chemical Exposure and Health Research Team and [3]Research Team for Health Effects of Air Pollutants, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-0053, Japan and [4]Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Inage, Chiba 263-8522, Japan

[5]To whom correspondence should be addressed
Email: ybaoki@nies.go.jp

Exposure to diesel exhaust (DE) is known to cause lung tumors in rats. To clarify the mutagenicity of DE, we estimated mutant frequency (MF) and determined the mutation spectra in rat lung after exposure to DE using *lambda/lacI* transgenic rats (Big Blue® system). Male Big Blue® rats (6 weeks old) were exposed for 4 weeks to 1 or 6 mg/m^3 DE, which contains suspended particulate matter. Control rats were maintained in filtered clean air. After exposure to 6 mg/m^3 DE, MF in lung was 4.8-fold higher than in control rats ($P < 0.01$), but no increase in MF was observed in rats exposed to 1 mg/m^3 DE. Sixty-nine mutants were identified after exposure to 6 mg/m^3 DE. The major mutations were A:T→G:C (18 mutations) and G:C→A:T (19 mutations) transitions. Remarkably, G→T transversion of the *lacI* gene at site 221 was a hot-spot induced by exposure to DE, and there were complex mutations in which multiple mutations occurred in a single mutant, especially in the rats exposed to 6 mg/m^3 DE. DNA adducts formed by DE were analyzed using a ^{32}P-post-label TLC method and the amount of 8-hydroxydeoxyguanosine (8-OHdG) was measured using HPLC. Relative adduct level and amount of 8-OHdG were significantly increased in the rats exposed to 6 mg/m^3 DE compared with the controls (3.0- and 2.2-fold, respectively; $P < 0.01$). The level of cytochrome P450 1A1 mRNA was shown by northern blot analysis to be significantly increased in the lungs of rats exposed to 6 mg/m^3 DE (5.5-fold; $P < 0.01$). These results indicate that DE causes lesions in genomic DNA and acts as a mutagen in rat lung.

Introduction

Vehicles powered by diesel engines are a major source of suspended particulate matter (SPM), which is a suspected cause of lung cancer and allergic respiratory disease including bronchial asthma (1,2). These diseases are still serious human health problems especially in large cities in recent years.

It is well known that diesel exhaust (DE) contains various potent carcinogens and mutagens, such as polycyclic aromatic hydrocarbons (PAHs) and nitrated PAHs, on diesel exhaust particles (DEP) (3,4). Some of these compounds in DE are regarded as pulmonary carcinogens in animals (5–8). A two-stage model of carcinogenesis postulates that PAHs released from DEP generate DNA adducts which cause mutations in oncogenes and tumor suppressor genes and act as initiators of carcinogenesis. DNA adducts were identified in rats after both short-term (12 weeks) (9) and long-term (30 months) (10) exposure to DE, and the level of DNA adducts was shown to be higher in lung tumor tissues than in normal tissues after chronic exposure to DE (11). Mutations have been detected in an oncogene and a tumor suppressor gene in tumors produced by DE and DEP, but these results are controversial. For example, point mutations in codons 12 and 13 of the k-*ras* oncogene were detected in rat lung adenomas and adenocarcinomas produced by intratracheal instillation of DEP (12). But mutations have not occurred in the *p53* tumor suppressor gene and the k-*ras* oncogene in rat lung squamous cell carcinomas (SCCs) and adenosquamous carcinomas (ACs) induced by chronic exposure to DE (13). On the other hand, DEP contains trace elements such as zinc, iron, copper, silicon and chromium (3) which are thought to catalyze production of oxygen free radicals such as superoxide ($O_2^{-}\cdot$) and hydroxyl radical (OH·) by autooxidation of quinone and polyphenol compounds (14). Therefore, oxygen free radicals generated by DEP may cause mutations in lung. In fact, oxidative damage to DNA, e.g. occurrence of 8-hydroxydeoxyguanosine (8-OHdG), was shown to increase in mouse lung during tumorigenesis by DEP (15,16). However, the mutagenic potential of DE has not been estimated.

In this study, we attempted to analyze the mutant frequency (MF) and mutation spectra (MS) after exposure to DE at concentrations of 1 and 6 mg SPM/m^3 of a transgenic rat, Big Blue® rat, which was developed for the detection of MF and MS. In this transgenic rat, the *lacI* genes of *Escherichia coli* carried on a *lambda* phage shuttle vector are integrated into chromosomal DNA. Mutations occurring in integrated *lacI* genes are assayed after the *lacI* genes are rescued into host *E.coli*. Similar transgenic mice containing *lacI* genes and *lacZ* genes named Big Blue® mouse and Muta™Mouse, respectively, have been developed and used to determine mutant frequency and mutation spectra of various mutagens, e.g. ethyl-*N*-nitrosourea (17), aflatoxin B_1 (18) and benzo[*a*]pyrene (B[*a*]P) (19). Using these transgenic animal models, it became possible to quantitatively detect the mutations on chromosomal DNA in the somatic cells of any organs.

Formation of DNA adducts and amounts of 8-OHdG in the lungs of Big Blue® rats exposed to DE were also analyzed. We present evidence that after exposure to DE the mutation frequency of the *lacI* gene was elevated in lung along with an increase in the amounts of DNA adducts.

Materials and methods

Animals and exposure to DE

Five-week-old male Big Blue® transgenic F344 rats were obtained from Stratagene (La Jolla, CA). The rats were maintained in a semi-clean air-

Abbreviations: B[*a*]P, benzo[*a*]pyrene; CYP, cytochrome P450; DE, diesel exhaust; DEP, diesel exhaust particles; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MF, mutant frequency; MS, mutation spectra; 8-OHdG, 8-hydroxydeoxyguanosine; PAHs, polycyclic aromatic hydrocarbons; RAL, relative adduct level; ROS, reactive oxygen species; SPM, suspended particulate matter.

Table I. Mutant frequencies in the lung of Big Blue® rats after exposure to DE or clean air

Concentration of DE (mg SPM/m^3)	ID of animals	No. of plaques		Mutant frequency ($\times 10^{-5}$)	Average mutant frequency ± SE ($\times 10^{-5}$)
		Mutant	Total		
Clean air	C/1	2	256051	0.78	
	C/2	2	248760	0.80	
	C/3	3	260047	0.77	
	C/4	2	258938	0.88	0.88 ± 0.091*
	Total	9	1023796		
1	1/1	3	311273	0.96	
	1/2	2	201563	0.99	
	1/3	2	207498	0.96	
	1/4	3	221175	1.35	
	1/5	2	218956	0.91	1.03 ± 0.081*
	Total	12	1160465		
6	6/1	10	220950	4.52	
	6/2	10	237242	4.22	
	6/3	9	221225	4.06	
	6/4	9	220020	4.09	
	6/5	10	227800	4.38	4.25 ± 0.087
	Total	48	1127237		

*$P < 0.01$ as compared with 6 mg/m^3 DE as SPM.

conditioned room at 24–26°C and 55–75% humidity with a 14–10 h light–dark cycle for 7 days before use. Exposure to DE (12 h/day, 7 days/week) was performed in chambers as described previously (20,21). Briefly, DE was generated by a light duty (2740 cc) four-cylinder diesel engine (A4JB1-type, Isuzu Automobile Company, Tokyo, Japan). The engine was operated using standard diesel fuel and was controlled by a computer to a speed of 1500 r.p.m. under a load of 10 torque (kg/m). Concentrations of fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene in DEP used in the present study were 130, 46.2, 22.0, 57.0, 28.2, 7.30, 2.00, 162, 0.40, 0.27 and 0.42 pmol/mg, respectively. Five animals each were exposed to DE at concentrations of 1 or 6 mg/m^3 as SPM (1 mg/m^3 DE group and 6 mg/m^3 group, respectively) for 4 weeks. Four animals were maintained in filtered clean air (control group). The animals were killed 3 days after the last day of exposure. The lobes of lungs were carefully removed from main bronchous and excised livers were separated from blood vessels and connective tissues. The tissues were frozen at –80°C, were crushed into small pieces homogeneously and stored at –80°C.

Tissues from all of the animals were subjected to mutation analysis and detection of DNA adducts, 8-OHdG and mRNAs. All analyses were carried out on coded samples.

LacI transgenic rat assay

Chromosomal DNA was extracted from lung and liver using the RecoverEase™ DNA isolation kit (Stratagene). The *lambda* shuttle vector was recovered as viable phage from chromosomal DNA to *in vitro* in *lambda* packaging extracts (Transpack™, Stratagene). *LacI* mutations were tested on *E.coli* (SCS-8 strain, Stratagene), which contains the remaining carboxyl terminal portion of the *lacZ* gene, as described previously (17,18). *E.coli* cells were infected with the *lambda* shuttle vectors and plated on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Stratagene). Mutants were identified as blue plaques. They were recovered and stored at 4°C in SM buffer [50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl, 8 mM $MgSO_4$ and 0.01% gelatin] until sequence analysis. The control mutants (CM-0 and CM-1 of the Big Blue® plaque color controls) were purchased from Stratagene.

Nucleotide sequence determination

Mutations in the *lacI* gene were determined by the cycle sequencing method (22). The total coding region of *lacI* and its promoter were amplified by PCR using *Taq* DNA polymerase (Promega, Madison, WI) with the following primers, 5′-GACACCATCGAATGGTGCAA-3′ (Y1) for the forward direction and 5′-TTCCACACATACGAGCC-3′ (Y2) for the reverse direction (23). Each PCR cycle consisted of denaturing at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 2 min. Forty cycles were performed. PCR fragments of the genes were directly cloned in PCR II vector (Invitrogen, San Diego, CA). Full length *lacI* PCR product was purified using a S.N.A.P.™ Miniprep kit (Invitrogen) and the full length of the *lacI* gene was sequenced using a Model 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Analysis by a ^{32}P-post-labeling TLC method of DNA adducts formed by PAHs

DNA was isolated from frozen lung essentially according to the procedure of Randerath *et al.* (24). Minced tissues were homogenized with a Polytoron homogenizer in 15 mM sodium citrate buffer containing 0.14 M NaCl (pH 7.0). The homogenate was centrifuged at 12 000 *g* for 20 min at 4°C, and the resulting pellet was suspended in 10 mM Tris–HCl buffer (pH 7.0) containing 1 mM EDTA and 1 M NaCl. After the suspension was mixed with 10% (w/v) SDS solution (0.1 ml/g tissue), it was digested with proteinase K (5 U/g; Merck, Darmstadt, Germany) at 37°C for 40 min and then shaken well with chloroform/isoamyl alcohol (24:1 v/v). The aqueous layer was mixed with an equal volume of 2-ethoxyethanol and kept at –20°C overnight. The precipitate containing DNA was removed with a hooked glass rod and dissolved in TE buffer [0.01 M Tris–HCl buffer (pH 7.0) containing 0.001 M EDTA]. RNA and protein in the precipitate were digested with RNase A (9 U/g; Nippongene, Toyama, Japan) and RNase T1 (1600 U/g; Worthington Biochemical Co., Freehold, MO) at 37°C for 40 min followed by proteinase K (3 U/g) for 40 min. The solution was repeatedly washed with 2 vol chloroform/isoamyl alcohol (24:1 v/v) for 15 min followed by centrifugation at 20 000 *g* for 20 min at 4°C until the ratios of absorbance (A) at 260/280 and 260/230 nm of the aqueous layer were greater than 1.8 and 2.0, respectively.

DNA in the aqueous layer was precipitated by adding 0.1 vol 5 M NaCl and an equal volume of cold 2-ethoxyethanol. After standing for a few minutes the precipitate was removed and washed twice with ethanol, once with ethanol/ether (50:50) and twice with ether. The DNA was dried and stored at –20°C.

DNA adducts were detected by a ^{32}P-post-labeling TLC method (25–28) after treatment of the DNA with nuclease P1 (Wako, Tokyo, Japan). DNA (10 μg) was hydrolyzed to 2′-deoxyribonucleotide 3′-monophosphates (dNp) at 37°C for 3 h with micrococcal nuclease (0.6 U; Worthington Biochemical Co.) and spleen phosphodiesterase (0.01 U; Worthington Biochemical Co.) in 0.1 M sodium succinate buffer (pH 6.0) containing 0.05 M $CaCl_2$. The digest (10 μl) was mixed with nuclease P1 (4 mg/ml) in 0.3 M sodium acetate (pH 5.3) containing 1 mM $ZnCl_2$. The reaction was stopped by adding 3 μl of 0.5 M Tris-base.

After adding 0.85 MBq of [γ-^{32}P]ATP (111 Tbq/mmol; Amersham, Japan) and T4 polynucleotide kinase (5 U; Takara, Kyoto, Japan), the mixture (15 μl) was incubated at 37°C for 30 min for ^{32}P-post-labeling. Unreacted [γ-^{32}P]ATP was degraded by addition of a solution of potato apyrase (2.5 μl, 20 U/mg; Sigma, St Louis, MO) and incubation at 37°C for 45 min.

The mixture was applied to a pre-washed PEI-TLC plate (10×20 cm; Mashery-Nagel, Dure, Germany) which was developed with 2.3 M sodium phosphate buffer (pH 6.0) (the first dimension, D1) for 14 h to separate the adducts from normal nucleotides and ATP. A piece of plate around the application point was cut off and was attached to another PEI-TLC plate (10×10 cm) which was developed with 3.5 M lithium formate (pH 3.5) containing 8.5 M urea (D2). The plate was turned 90° and developed with 0.5 M Tris–HCl buffer (pH 8.0) containing 8.5 M urea and 0.8 M LiCl (D3). It was washed with 1.7 M sodium phosphate buffer (pH 6.0) (D4).

^{32}P-labeled DNA adducts on the TLC plate were visualized and their

Table II. Summary of mutations detected in the present study

ID of animals[a] and clone no.	Base no.	Sequence context	Base change or type of mutations	Amino acid change
C/1-1	92	CGC→GC	deletion	frameshift
	719	ATG→AATG	insertion	frameshift
C/1-2	93	CGC→CAC	G→A	Arg→His
C/2-1	485	CAG→TAG	G→T	Gln→stop
C/2-2	886	TTA→TTT	A→T	Leu→Phe
C/3-1	955	CAG→CA	deletion	frameshift
C/3-2	308	GTC→ATC	G→A	Val→Ile
C/3-3	464	TTA→GTA	T→G	Leu→Val
C/4-1	221	GGC→TGC	G→T	Gly→Cys
C/4-2	1086	GTT→GAT	T→A	Val→Asp
1/1-1	631	CAT→CTGGCAT	insertion	frameshift
1/1-2	-29	ACC→GCC	A→G	-
1/1-3	442	GCT→GC	deletion	frameshift
1/2-1	485	CAG→TAG	C→T	Gln→stop
	613	CGT→CGC	T→C	Arg→Arg
	1111	TGA→TGG	A→G	-
1/2-2	ND**			
1/3-1	-2	GAT→GAC	T→C	-
	192-194	CAAC→C	deletion	-
	906	TTT→TGT	T→G	Phe→Cys
	1003	AAA→AGA	A→G	Lys→Arg
1/3-2	93	CGC→CAC	G→A	Arg→His
1/3-3	4	GGA→GAG	A→G	-
1/4-1	221	GGC→TGC	G→T	Gly→Cys
1/4-2	534	CTG→CCG	T→C	Leu→Pro
1/4-3	765	AAC→AGC	A→G	Asn→Ser
1/5-1	118	GTT→GTC	T→C	Val→Val
1/5-2	877	ATC→ATG	C→G	Ile→Met
6/1-1	-8	TGA→TGG	A→G	-
	56	GCA→ACA	G→A	Ala→Thr
	85	ACC→ACT	C→T	Thr→Thr
	625	GCT→GCC	T→C	Ala→Ala
	804	GAG→GGG	A→G	Glu→Gly
6/1-2	955	CAG→CA	deletion	frameshift
6/1-3	90*	TCC→TAC	C→A	Ser→Tyr
	206	CAG→TAG	C→T	Gln→stop
6/1-4	716	ATG→CATG	insertion	frameshft
6/1-5	90*	TCC→TAC	C→A	Ser→Tyr
	1138	GCT→GCA	T→A	-
6/1-6	221*	GGC→TGC	G→T	Gly→Cys
6/1-7	886	TTA→TTT	A→T	Leu→Phe
	221*	GGC→TGC	G→T	Gly→Cys
6/1-8	723	ATG→ATTG	insertion	frameshift
	92	CGC→GC	deletion	frameshift
6/1-9	360	GTG→GCG	T→C	Val→Ala
6/1-10	118	GTT→GTC	T→C	Val→Val
6/2-1	102	AAC→AGC	A→G	Asn→Ser
	221*	GGC→TGC	G→T	Gly→Cys
	1012	ACC→ACT	C→T	Thr→Thr
6/2-2	221*	GGC→TGC	G→T	Gly→Cys
6/2-3	ND			
6/2-4	631	CAT→CTGGCAT	insertion	frameshift
6/2-5	-37	TGG→CGG	T→C	-
	329	CGA→TGA	C→T	Arg→stop
6/2-6	1078	GCA→GCG	A→G	Ala→Ala
	382	CGA→TGA	C→T	Arg→stop
6/2-7	1008	AAA→AGA	A→G	Lys→Arg
	906	TTT→TGT	T→G	Phe→Cys
6/2-8	192-194	CAAC→C	deletion	frameshift
6/2-9	65	GCC→ACC	G→A	Ala→Thr
6/2-10	429	ATT→ACT	T→C	Ile→Thr
	381	CGC→CAC	G→A	Arg→His
6/3-1	722	CAA→CAAA	insertion	frameshift
6/3-2	891	ACC→ACT	C→T	Thr→Thr
6/3-3	66	GCC→GTC	C→T	Ala→Val
6/3-4	983	TTG→CTG	T→C	Leu→Leu
6/3-6	221	GGC→TGC	G→T	Gly→Cys
6/3-7	937	CGC→CGT	C→T	Arg→Arg
	886	TTA→TTT	A→T	Leu→Phe
6/3-8	182	GTC→ATG	G→A	Val→Met
	444	GCC→GGC	C→G	Ala→Gly
6/3-9	918	GGG→GAC	G→A	Gly→Asp
6/4-1	601	TCT→TCC	T→C	Ser→Ser
	704	TTT→ATT	T→A	Phe→Ile
6/4-2	685	GAC→GA	deletion	frameshift
6/4-3	221	GGC→TGC	G→T	Gly→Cys
	449	ACT→GCT	A→G	Thr→Ala
6/4-4	892	ACC→ACT	C→T	Thr→Thr
6/4-6	51	GAT→GGT	A→G	Asp→Gly
	272	GCG→CCG	G→C	Ala→Pro
6/4-7	329	CGA→TGA	C→T	Arg→stop
6/4-8	221	GGC→TGC	G→T	Gly→Cys
6/4-9	-29	ACC→GCC	A→G	-
	202	GGC→GG	deletion	frameshift
6/5-1	202	GGC→GG	deletion	frameshift
6/5-2	329	CGA→TGA	C→T	Arg→stop
6/5-3	296	CTG→TTG	C→T	Leu→Leu
6/5-4	221	GGC→TGC	G→T	Gly→Cys
6/5-5	847	TAC→TAG	C→G	Tyr→stop
	1031	CAA→TAA	C→T	Gln→stop
6/5-6	-44	ACC→ACA	C→A	-
	49	TAC→TAA	C→A	Tyr→stop
6/5-7	886	TTA→TTT	A→T	Leu→Phe
6/5-8	723	ATG→ATTG	insertion	frameshift
6/5-9	847	TAC→TAG	C→G	Tyr→stop
	1169	TAT→CAT	T→C	-
6/5-10	82	CAG→CAT	G→T	Glu→His
	95	GTG→ATG	G→A	Val→Met

[a]See Table I.

*Some identical mutations at the same site in individual mutants recovered from one animal were counted as one mutation. Independence of these mutations is uncertain.

**ND indicates the clones in which the sequence of the full length of the gene was not able to be determined.

radioactivities quantified with a bioimaging analyzer (Bas 2000, Fuji Film, Tokyo, Japan). The levels of DNA adducts were estimated by the relative adduct level (RAL) which is defined as (25–28):

$$\text{RAL} = \text{c.p.m. in adduct nucleotides}/(\text{c.p.m. in total nucleotides} \times \text{dilution factor})$$

To determine the radioactivity of the total nucleotides, 5 µl of the T4 polynucleotide kinase treated mixture was spotted on a PEI-TLC plate and radioactivity around the application point was measured after the plate was developed with 1.7 M sodium phosphate buffer (pH 6.0).

Analysis of 8-OHdG

A modification of Marmur's method (29) was used for extracting DNA from lung (16). DNA was digested to deoxynucleotides according to the procedure of Ichinose *et al.* (16,30). The content of 8-OHdG in the digested DNA was measured by HPLC with an electrochemical detector (CoulochemII, Esa, Tokyo, Japan) (15,30,31). The total amount of deoxyguanosine (dG) was simultaneously detected at 290 nm with a UV detector (UV-8020; Tosho, Tokyo, Japan). The content of 8-OHdG in DNA was expressed as the ratio of 8-OHdG to total dG.

Northern blot analysis

Total RNA was isolated from lung by the acid guanidium–thiocyanate phenol–chloroform extraction method (32) using Isogen reagent (Nippongene, Toyama, Japan). Ten micrograms of total RNA was denatured in glyoxal and dimethyl sulfoxide, separated on a 1% agarose gel and transferred to a nylon membrane (Hybond™ N; Amersham). After hybridization with ^{32}P-labeled probes as described below, radioactive bands were visualized and quantified with a bioimaging analyzer (Bas 2000). The expression level of the cytochrome P450 (CYP)1A1 mRNA in each lung sample was standardized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal marker. cDNA probes consisted of a 1.2 kb *Pst*I fragment of the murine CYP1A1 gene (ATCC #63006) and an 855 bp fragment of the GAPDH gene (kindly provided by Drs T.Fujii and Y.Mitsui). A cDNA probe for CYP1A2 was isolated by the RT–PCR method (33,34). It was generated by superscript reverse transcriptase (Gibco BRL, Gaithersburg, MD) with 10 µg of total RNA isolated from Wistar rat liver 72 h after administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (300 ng/kg) and oligo(dT) as a primer. cDNA was amplified by PCR using *Taq* DNA polymerase (Promega) with primers: forward, ACAGTCCAG-GAACACTATC; reverse, ATGAATCTTCCTCCTCTGCACCTT. The PCR cycle consisted of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 30 s. Forty cycles were performed. PCR fragments of the genes were directly cloned in PCRII vector (Invitrogen). After confirmation of the sequences of the inserts using an ALF™ DNA sequencer (Pharmacia Biotech, Tokyo, Japan), the probe was used for northern blot analysis. It did not hybridize to CYP1A1 mRNA.

Table III. DE-induced mutations in *lacI* gene in lung of Big Blue® rats

Mutation type	Base substitution	Mutations					
		Control		DE-induced			
		n	%	1 mg/m³		6 mg/m³	
				n	%	*n*	%
Transition	A:T→G:C	0	0	9	52.9	18	26.0
	G:C→A:T	3(1)[a]	30	2(0)[a]	11.8	19(3)[a]	27.5
Transversion	G:C→T:A	1	20	1	5.9	9	13.0
	G:C→C:G	0	0	1	5.9	5	7.2
	A:T→C:G	1	10	1	5.9	1	1.4
	A:T→T:A	2	20	0	0	6	8.7
Single base deletion		2	20	1	5.9	5	7.2
Multi base deletion	ACC	0	0	1	5.9	1	1.4
Single base insertion		1	10	0	0	4	5.8
Multi base insertion	TGGC	0	0	1	5.9	1	1.4
Total		10	100	17	100	69	100

[a]Data in parentheses are the numbers of mutations at CpG sites, where spontaneous deamination of methylcytosine induces G:C→A:T transition.

Statistics

Student's *t*-test was used for determining statistical significance.

Results

LacI mutant frequencies and mutation spectra in lung of rats

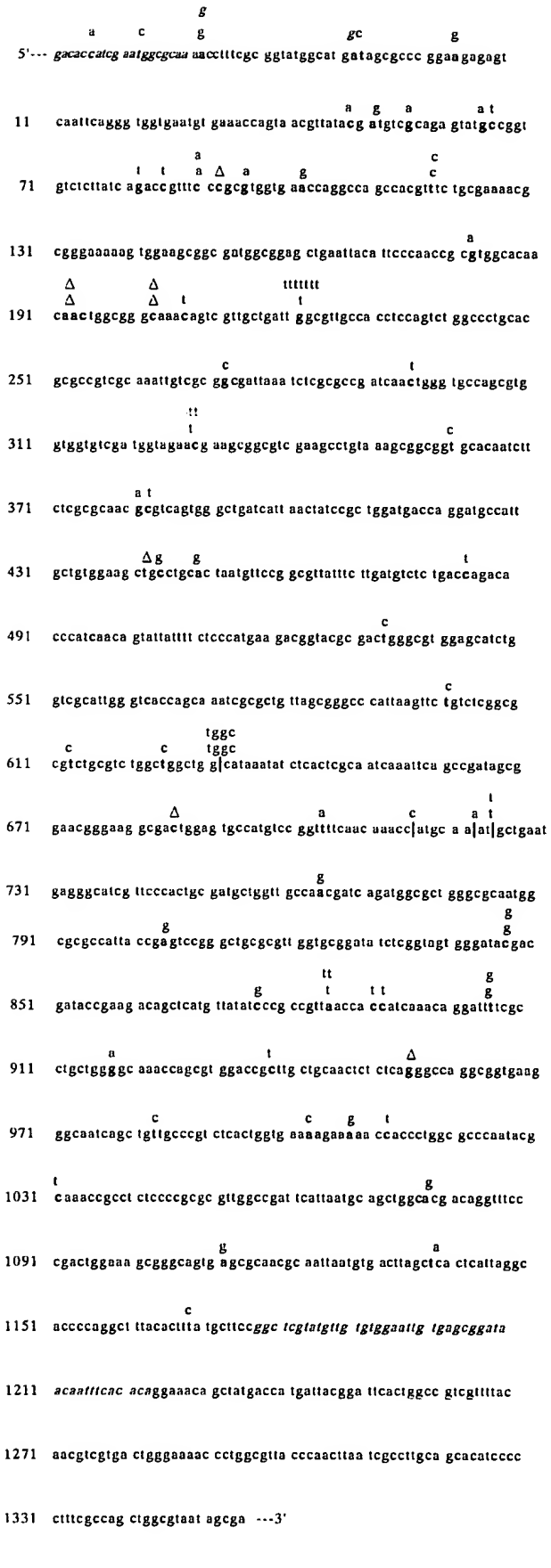
We estimated MFs of the *lacI* gene and determined the MS in the lungs of Big Blue® rats after exposure for 4 weeks to DE at concentrations of 1 and 6 mg/m³, which contained SPM. Control rats were maintained in filtered clean air. As shown in Table I, while only nine mutants were obtained from the control group, there were 12 and 48 mutants in the 1 mg/m³ DE group and 6 mg/m³ DE group, respectively. MF in the control rat lungs was estimated to be 0.88 ± 0.091 ($\times 10^{-5}$), while MF in the livers of the same control rats was estimated as 2.6 ± 0.047 ($\times 10^{-5}$, $n = 3$), and this value was the same as reported previously (35). MF in the lungs of the 6 mg/m³ DE group was significantly 4.8-fold higher than in the control group [average MF ± SE: 4.25 ± 0.087 versus 0.88 ± 0.091 ($\times 10^{-5}$); $P < 0.01$]. However, no significant difference in MF was observed between the 1 mg/m³ DE group and the control group (1.03 ± 0.081 versus 0.88 ± 0.091). In order to confirm the reliability of plating conditions, we plated the control mutants (CM-0 and CM-1 of the Big Blue® plaque color controls). In every experiment, we detected 15–25 mutants per 2500 non-mutant plaques, which was the same as described in the Big Blue® system supplier's instruction manual, indicating that the plating conditions were consistent among the experiments.

Mutations induced in the *lacI* gene of lung DNA are shown in Table II and the types of mutations found in the control and the DE-exposed lung tissues are summarized in Table III. Two mutants carrying a single mutation at site 221 of the *lacI* gene were recovered from one animal (ID of animal 6/2) but these were counted as one mutation. Ten independent mutations were identified in nine mutants of the control group. Two single base deletions (–C and –G) and one single insertion (+A) were found in the lung DNA of control rats. Seven single base substitution mutations (three G:C→A:T, one G:C→T:A, one A:T→C:G, two A:T→T:A mutations) were also found (Tables II and III), and one mutation was detected at CpG sites in the control group.

In the DE-exposed group, 17 and 69 different mutations were identified in 11 mutants of the 1 mg/m³ DE group and 47 mutants of the 6 mg/m³ DE group, respectively. However, two clones (1/2-2 and 6/2-3) could not be sequenced. In the 1 mg/m³ DE group, 14 single base substitutions, one single base deletion and one three-base deletion (–AAC) were found (Table III), whereas complex mutations, in which multiple mutations occurred in a single mutant, were detected in two mutants (clones 1/2-1 and 1/3-1; Table II). The major mutations (52.9%) were A:T→G:C transitions and 11.8% of the mutations were G:C→A:T transitions that occurred at non-CpG sites (Table III). In the 6 mg/m³ DE group, complex mutations were also detected in 20 mutants (clones 6/1-1, 6/1-3, 6/1-5, 6/1-7, 6/1-8, 6/2-1,6/2-5, 6/2-6, 6/2-7, 6/2-10, 6/3-7, 6/3-8, 6/4-1, 6/4-3, 6/4-6, 6/4-9, 6/5-5, 6/5-6, 6/5-9 and 6/5-10; Table II). The major mutations were G:C→A:T transitions (19 mutations, 27.5%), including three mutations at CpG sites, and A:T→G:C transitions (18 mutations including 10 independent T→C substitutions and eight independent A→G substitutions, 26.0%), while nine mutations were G:C→T:A transversions; five were G→T and four were C→A. Five single base deletions (–G and –C), one three-base deletion (–ACC) and four single base insertions (+C and +A) were detected (Table III). Two four-base insertion (+TGGC) mutations occurred at positions 631 in either the 1 or 6 mg/m³ DE group. This type of mutation is reported to be frequent in the *lacI* gene (36,37). The distribution of the mutations in the *lacI* gene in rat lung exposed to DE is shown in Figure 1. The most frequent mutations occurred at site 221 of the gene (one animal in the 1 mg/m³ DE group and all five animals in the 6 mg/m³ DE group, G→T). Site 221 was a hot-spot for the mutations (G→T) caused by exposure to DE, while several mutations were detected in two or three independent mutants.

DNA adduct formation induced by DE

To clarify how the mutant frequency in lung DNA was increased after exposure to 6 mg/m³ DE, we analyzed formation of DNA adducts in lung, liver and nasal mucosa using a ^{32}P-post-label TLC method which is suitable for detecting adducts produced from PAHs. Two major adducts and one minor adduct were detected in lung tissues (Figure 2). Total RAL of these three adducts in lung is shown in Table IV. The total



RAL in lung from the 6 mg/m^3 DE group was significantly higher than that in the control group and the 1 mg/m^3 DE group (3.02- and 1.48-fold, respectively; Table IV). In liver and nasal mucosa, no significant changes were observed in the amount of DNA adducts in both the 1 and 6 mg/m^3 DE groups (data not shown). To estimate DNA damage caused by reactive oxygen species (ROS) generated by DE, we measured the amount of 8-OHdG in rat lung after exposure to DE for 4 weeks (Table IV). The relative amounts of 8-OHdG (8-OHdG/dG) were significantly elevated after exposure to DE for 4 weeks and increased with increasing concentration of DE. The levels of 8-OHdG in the 1 mg/m^3 DE group and 6 mg/m^3 DE group were 169 and 216%, respectively, of that of the control (Table V).

Induction of CYP1A1 mRNA after exposure to DE

Since formation of DNA adducts from PAHs is known to be catalyzed by CYP1A1 and CYP1A2, expression of the CYP1A1 and CYP1A2 genes caused by exposure to DE was determined by northern blot analysis. As shown in Figure 3, the relative level of CYP1A1 mRNA in lung of the 6 mg/m^3 DE group was 5.8-fold higher than in the control group ($P < 0.01$) and was also significantly higher than in the 1 mg/m^3 DE group. The CYP1A1 mRNA level was not significantly elevated in the 1 mg/m^3 DE group because its variability was large. The CYP1A2 mRNA was not detectable in lungs from both of the groups exposed to DE and from the control group (data not shown). As an internal marker, the amounts of GAPDH mRNA were identical in the control and DE groups (data not shown).

Discussion

DE, as well as DEP, cause pulmonary cancer in experimental animals (38). For example, SCCs and ACs were induced in the lungs of F344 rats by inhalation of DE at a concentration of 6.33 ± 0.04 mg/m^3 for 24 months (16 h/day, 5 days/week) (13). These carcinomas were also induced in the lungs of rats after long exposure (18 months) to DE at a concentration of 6.5 mg/m^3 (38). Not only inhalation but also intratracheal instillation of DEP (5 mg/DEP once a week for 10 weeks) induced ACs in rat lung up to 30 months after the beginning of instillation (12). DE causes the formation of DNA adducts in the lungs of experimental animals (39) and DNA adducts were detected in the lymphocytes of workers driving diesel forklifts (40), suggesting that DE contributes to elevated DNA adduct formation in humans.

However, it has been unclear how DE causes mutations in the lungs of rats. In order to estimate the mutagenicity of DE before tumors arise, we analyzed MF and MS in Big Blue® rat lungs after 4 weeks of exposure to DE at a concentration that causes tumors (6 mg/m^3). Big Blue® rats are transgenic animals in which the *E.coli lacI* gene is integrated into chromosomal DNA as a monitor for detecting mutations. With such transgenic animals, it is possible to directly determine

Fig. 1. Overall distribution of the mutations detected in the *lacI* gene in rat lung exposed to DE. Summary of the mutations detected in 1 and 6 mg/m^3 DE groups listed in Table II. The mutations induced by DE are shown above the sequence. Numbers of letters above the sequence indicate the numbers of mutations. Δ (deletion), I (position of insertion), bold (gene mutations in the *lacI* gene). The primers are indicated in italics.

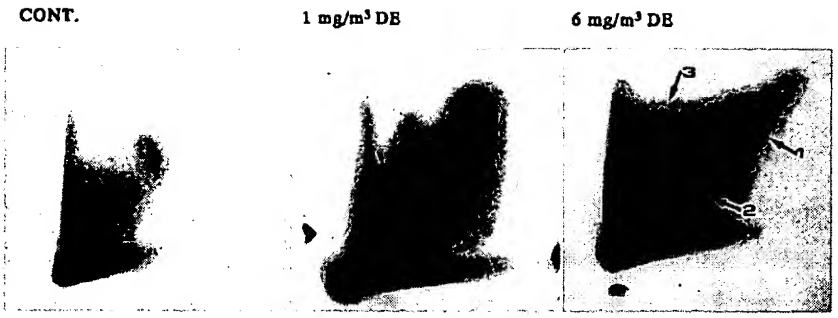


Fig. 2. Fingerprints of ^{32}P-post-labeled DNA adduct in lung of Big Blue® rats exposed to DE (1 or 6 mg/m^3 as SPM) or clean filtered air for 4 weeks. The adducts were separated on a TLC plate and analyzed with a bioimaging analyzer (Bas 2000) as described in Materials and methods. Numbers indicate the different spots obtained.

Table IV. DNA adduct levels (RAL) in lungs of control and Big Blue® rats exposed to DE (1 or 6 mg/m^3 DE as SPM) for 4 weeks

Concentration of DE (mg SPM/m^3)	*n*	Average of total RAL (per 10^8 nucleotides)	%
Control	4	14.65 ± 2.24**	100
1	5	22.89 ± 4.57*	148
6	5	47.84 ± 6.76	302

Data are shown as means ± SE.
**$P < 0.01$ as compared with 6 mg/m^3 DE as SPM.
*$P < 0.05$ as compared with 6 mg/m^3 DE as SPM.

Table V. Formation of 8-OHdG in Big Blue® rats lung after 4 weeks of exposure to filtered air or DE (1 and 6 mg/m^3 DE as SPM)

Concentration of DE (mg SPM/m^3)	8-OHdG/dG (10^{-5})	%
Control	3.53 ± 0.63	100
1	5.96 ± 0.65*	169
6	7.64 ± 0.49**	216

Big Blue® rats were exposed to 1 or 6 mg/m^3 DE as SPM or filtered clean air (control) for 4 weeks. Data are shown as means ± SE.
**$P < 0.01$ as compared with control.
*$P < 0.05$ as compared with control.

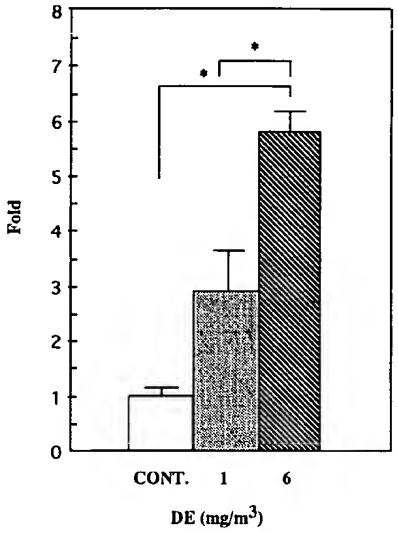


Fig. 3. Relative expression level of CYP1A1 mRNA in lung of Big Blue® rats exposed to DE (1 or 6 mg/m^3 as SPM) or clean filtered air for 4 weeks. The expression level of CYP1A1 mRNA in each lung sample was standardized to that of GAPDH mRNA as an internal marker as described in Materials and methods. Data are shown as means ± SE. *$P < 0.01$.

mutations on animal tissue chromosomal DNA induced by environmental mutagens.

As shown in Table I, a significant increase in MF was observed in rat lungs after exposure to 6 mg/m^3 DE for 4 weeks (4.8-fold higher than in the controls), but MF was not significantly elevated after exposure to 1 mg/m^3 DE. These results suggest that mutations accumulated on lung DNA initiate pulmonary cancer in the rats.

Mutations induced by DE are thought to be caused by formation of DNA adducts. DEP contains various potent carcinogens and mutagens, such as PAHs and nitrated PAHs (3,4), which induce the formation of DNA adducts. Diesel particle extract was shown to produce DNA adducts *in vitro* with rat liver S9 mixture, and *in vivo* in rat lungs after 24 months of exposure to DE (7.5 mg/m^3) (39). An unrepaired DNA adduct causes a mismatch of a base pair in DNA resulting in a gene mutation (41,42). A cell bearing a mutated proto-oncogene(s) is thought to develop into a tumor through the processes of promotion and progression (43,44). For example, B[*a*]P induces a G→T base transversion in H-*ras* genes (45) which converts *ras* proto-oncogenes to their active form (46). In the present study, the amount of DNA adducts was significantly elevated in rat lungs after exposure to 6 mg/m^3 DE for 4 weeks but not after exposure to 1 mg/m^3 DE (Table IV). Bond *et al.* (47) reported that the amount of DNA adducts in rat alveolar type II cells after exposure to 6.2 mg/m^3 DE for 12 weeks was 5-fold higher than in control rats. These results suggest that the formation of DNA adducts causes the increase in MF in rat lungs after exposure to DE.

Formation of DNA adducts was catalyzed by CYP1A1 and CYP1A2, which are inducible by PAHs (34,48). CYPs oxidize PAHs to reactive electrophilic metabolites which bind to DNA bases to form DNA adducts. Thus, we analyzed the induction of CYP1A1 and CYP1A2 mRNAs in the lungs of Big Blue® rats exposed to DE. As shown in Figure 3, the level of CYP1A1 mRNA was significantly elevated in lung by exposure to 6 mg/m^3 DE ($P < 0.01$), but not by exposure to 1 mg/m^3 DE. These results suggest that the formation of DNA adducts in rat lung was stimulated by CYP1A1 which was induced by 6 mg/m^3 DE. On the other hand, CYP1A2 mRNA was not detectable in lung after exposure to 6 mg/m^3 DE (data not shown). It is unclear how CYP1A1 mRNA was induced by DE, but DE may contain a specific inducer of CYP1A1 mRNA.

β-Naphthoflavone is a good example of a specific inducer of CYP1A1 but not of CYP1A2 in rat lung after intraperitoneal administration, and the formation of B[*a*]P–DNA adducts was stimulated in the lungs of rats which had been given β-naphthoflavone (49). Oxidative gas, as well as PAHs, induces CYP1A1 and/or CYP1A2. Takahashi and Miura (50) reported that inhalation of nitrogen dioxide elevates rat lung 7-ethoxycoumarin *O*-deethylase activity, which is catalyzed by CYP1A1 and CYP1A2.

Not only PAHs but ROS, such as superoxide ($O_2^{-}\cdot$) and hydroxyl radicals (OH·) generated by DEP, also cause mutations (36). The hydroxyl radical produces 8-OHdG and other hydroxylated deoxynucleotides, which induce DNA changes prior to tumorigenesis in mice (16). 8-OHdG was shown to be induced in mouse lung by ROS generated by DEP (30). Ichinose *et al.* (15) reported that the amount of 8-OHdG in lung produced by instillation of washed DEP was significantly correlated with the development of lung tumors. In our study, the content of 8-OHdG in the lungs of Big Blue® rats after exposure to 6 mg/m^3 DE for 4 weeks was significantly elevated (2.16-fold, $P < 0.01$; Table V). These results suggest that the increase in DNA damage induced by ROS also contributes to elevated MF in lungs.

In order to clarify the type of mutation generated in rat lung by DE, we analyzed MS in the *lacI* gene mutants obtained from Big Blue® rats. As shown in Table II and Figure 1, G→T transversions were detected at site 221 of one animal in the 1 mg/m^3 DE group and all five animals in the 6 mg/m^3 DE group. This mutation was the most frequent substitution in the present study, and was a hot-spot induced by exposure to DE, whereas it was not a major mutation (5.9% in the 1 mg/m^3 DE group and 13.0% in the 6 mg/m^3 DE group). G→T transversion is a major mutation caused by hydrodiol epoxides of PAHs, e.g. B[*a*]P, 7-methylbenz[*a*]anthracene and 5-methylchrysene dihydrodiol epoxides which selectively modify guanine residues (45,51–55). In Big Blue B6C 3F1 mice G:C→T:A transversions increased on *cII* transgenes of splenic T cells after B[*a*]P treatment (56). As well as PAH, 8-OHdG on DNA produced by ROS is also known to induce G:C→T:A transversions (57). Our results suggest that PAHs in DEP and/or ROS generated by DEP contribute to the increase in this type of transversion in rat lung after exposure to DE.

The most frequent mutations induced in DE exposed animals were A:T→G:C transitions (nine in the 1 mg/m^3 DE group and 18 in the 6 mg/m^3 DE group) and G:C→A:T transitions at non-CpG sites (two in the 1 mg/m^3 DE group and 16 in the 6 mg/m^3 DE group) but the inducer of the transitions has not been identified. Recently, C→T transitions were reported to be induced by 2-hydroxyadenosine, another hydroxylated nucleotide produced by ROS, in mammalian cells (58). Smith *et al.* (59) detected C→T transitions in exon 5–8 of the *p53* gene in two of 20 lung tumor samples from F344 rats treated with 1,6-dinitropyrene, a potent mutagen of DEP. Therefore, C→T substitutions detected in this study may be partly caused by ROS as well as nitroarenes, and are possibly associated with mutations of the *p53* gene which contribute to various types of cancer in rodents and humans.

As shown in Table II, it was remarkable that two to five mutations in a single mutant (complex mutations) were detected especially in rats exposed to 6 mg/m^3 DE. The formation of complex mutations has not been well documented. Mullin *et al.* (60) observed complex mutations in mouse lung after benzene inhalation and Akiyama *et al.* (61) detected them in a UV-irradiated rat fibroblast cell line. The complex mutations observed in this study may have been caused by multiple DNA adducts formed by various chemicals in DE.

Lung tissue injury caused by oxidative stress is known to accelerate the turnover of cells. For example, exposure of rats to oxidative gas decreases the turnover time of alveolar type II cells from 29 days, in normal rats, to 3 days (62), and rat lung tissue injury increases in the level of transforming growth factor α and stimulates the proliferation of alveolar type II cells (63). In the case of DE, pathologic alterations accompanied by cell proliferation and inflammation, e.g. Type II cell hyperplasia, squamous epithelial metaplasia and fibrosis, were also induced after long exposure (64). Therefore, we decided to use a shorter expression time to avoid further clonal expansion of mutated cells which continues after exposure to DE. In the present study, we adopted 3 days for the expression time. Adequate expression time has not been established for lungs and more studies for determining it are needed.

We demonstrate here that exposure of rat lung to 6 mg/m^3 DE increases MF and this increase in mutations, including complex mutations, might cause pulmonary cancer in F344 rats. Transitions were major mutations in DE-exposed animals and C→T transitions seem to be partly caused by ROS, but further studies are required to understand how DE causes the transition mutations shown in Table III. Such studies could reveal the mechanism of DE carcinogenesis. It is already known from bacterial tests, e.g. the Ames test (65) that nitroarenes in DEP are major mutagens. However, information about the mutagenicity of nitroarenes in animal tissues is very limited and should be analyzed using the Big Blue® system. Formation of DNA adducts has been used for the assessment of exposure to environmental mutagens and we have already detected DNA adducts in the lungs of rats exposed to urban air (H.Sato and Y.Aoki, unpublished data). It is difficult to estimate the total mutagenicity of various chemicals in the environment to which animals and humans are exposed. Mutation analysis using transgenic animals to detect mutagens should contribute to understanding the mechanism of carcinogenesis caused by environmental pollutants.

Acknowledgements

We would like to thank Dr Hiroshi Seto (Tokyo Metropolitan Research Laboratory of Public Health) for the measurement of 8-OHdG, Dr Kazuichi Hayakawa (Kanazawa University) for the measurement of PAHs and nitrated PAHs in DEP, and Dr Takamichi Ichinose (Oita University of Nursing and Health Sciences) for his critical comments on this study. H.S. and Y.A. also thank Drs Chiharu Tohyama and Gen Ohi (National Institute for Environmental Studies) for their continued encouragement.

References

1. Murasaka,M., Suzuki,S., Koizumi,K., Takafuji,S., Miyamoto,T., Ikemori,R. and Tokiwa,H. (1986) Adjuvant activity of diesel-exhaust particulates for the production of IgE antibody in mice. *J. Allergy Clin. Immunol.*, **77**, 616–623.
2. Takafuji,S., Suzuki,S., Koizumi,K., Tadokoro,K., Miyamoto,T., Ikemori,R. and Muranaka,M. (1987) Diesel-exhaust particulates inoculated by the intranasal route have an adjuvant activity for IgE production in mice. *J. Allergy Clin. Immunol.*, **79**, 639–645.
3. McClellan,R.O. (1987) Health effects of diesel exhaust particles. *Ann. Rev. Pharmacol. Toxicol.*, **27**, 279–300.
4. Hamada,K.P., Yamauchi,T., Ohnishi,M., Hisamatsu,Y. and Ishii,T. (1983) Detection and average content levels of carcinogenic and mutagenic compounds from the particulates on diesel and gasoline engine mufflers. *Environ. Int.*, **9**, 335–342.
5. Brightwell,J., Fouillet,X., Cassano-Zoppi,A.L., Gatz,R. and Duchosal,F.

(1986) Neoplastic and functional changes in rodents after chronic inhalation of engine exhaust emissions. *Dev. Toxicol. Environ. Sci.*, **13**, 471–485.
6. Stober,W. (1986) Toxicological effects of emissions from diesel engines. *Dev. Toxicol. Environ. Sci.*, **13**, 9–11.
7. Mauderly,J.L., Jones,R.K., McClellan,R.O., Henderson,R.F. and Griffith,W.C. (1986) Carcinogenicity of diesel exhaust inhaled chronically by rats. *Dev. Toxicol. Environ. Sci.*, **13**, 397–409.
8. Iwai,K., Udagawa,T., Yamagishi,M. and Yamada,H. (1986) Long-term inhalation studies of diesel exhaust on F344 SPF rats. Incidence of lung cancer and lymphoma. *Dev. Toxicol. Environ. Sci.*, **13**, 349–360.
9. Bond,J.A., Mauderly,J.L. and Wolff,R.K. (1990) Concentration and time-dependent formation of DNA adducts in lungs of rats exposed to diesel exhaust. *Toxicology*, **60**, 127–135.
10. Bond,J.A., Wolff,R.K., Harkema,J.R., Mauderly,J.L., Henderson,R.F., Griffith,W.C. and McClellan,R.O. (1988) Distribution of DNA adducts in the respiratory tract of rats exposed to diesel exhaust. *Toxicol. Appl. Pharmacol.*, **96**, 336–346.
11. Wong,D., Mitchell,C.E., Wolff,R.K., Mauderly,J.L. and Jeffrey,A.M. (1986) Identification of DNA damage as a result of exposure of rats to diesel engine exhaust. *Carcinogenesis*, **7**, 1595–1597.
12. Iwai,K., Higuchi,K., Udagawa,T., Ohtomo,K. and Kawabata,Y. (1997) Lung tumor induced by long-term inhalation or intratracheal instillation of diesel exhaust particles. *Exp. Toxic. Pathol.*, **49**, 393–401.
13. Swafford,D.S., Nikula,K.J., Mitchell,C.E. and Belinsky,S.A. (1995) Low frequency of alterations in p53, K-*ras* and mdm2 in rat lung neoplasms induced by diesel exhaust or carbon black. *Carcinogenesis*, **16**, 1215–1221.
14. Tappel,A.L. (1973) Lipid peroxidation damage to cell components. *Fed. Proc.*, **32**, 1870–1874.
15. Ichinose,T., Yajima,Y., Nagashima,M., Takenoshita,S., Nagamachi,Y. and Sagai,M. (1997) Lung carcinogenesis and formation of 8-hydroxydeoxyguanosine in mice by diesel exhaust particles. *Carcinogenesis*, **18**, 185–192.
16. Ichinose,T., Yamanushi,T., Seto,H. and Sagai,M. (1997) Oxygen radicals in lung carcinogenesis accompanying phagocytosis of diesel exhaust particles. *Int. J. Oncol.*, **11**, 571–575.
17. Kohler,S.W., Provost,G.S., Fieck,A., Kretz,P.L., Bullock,W.O., Sorge,J.A., Putman,D.L. and Short,J.M. (1991) Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. *Proc. Natl Acad. Sci. USA*, **88**, 7958–7962.
18. Dycaico,M.J., Stuart,G.R., Tobal,G.M., de Boer,J.G., Glickman,B.W. and Provost,G.S. (1996) Species-specific differences in hepatic mutant frequency and mutational spectrum among *lambda/lacI* transgenic rats and mice following exposure to aflatoxin B_1. *Carcinogenesis*, **17**, 2347–2356.
19. de Vries,A., Dolle,M.E., Broekhof,J.L., Muller,J.J., Kroese,E.D., van Kreijl,C.F., Capel,P.J., Vijg,J. and van Steeg,H. (1997) Induction of DNA adducts and mutations in spleen, liver and lung of XPA-deficient/*lacZ* transgenic mice after oral treatment with benzo[*a*]pyrene: correlation with tumor development. *Carcinogenesis*, **18**, 2327–2332.
20. Sagai,M., Saito,H., Ichinose,T., Kodama,M. and Mori,Y. (1993) Biological effects of diesel exhaust particles. I. *In vitro* production of superoxide and *in vivo* toxicity in mouse. *Free Radical Biol. Med.*, **14**, 37–47.
21. Ichinose,T., Furuyama,A. and Sagai,M. (1995) Biological effects of diesel exhaust particles (DEP). II. Acute toxicity of DEP introduced into lung by intratracheal instillation. *Toxicology*, **99**, 153–167.
22. Erfle,H.L., Walsh,D.F., Holcroft,J., Hague,N., de Boer,J.G. and Glickman,B.W. (1996) An efficient laboratory protocol for the sequencing of large numbers of *lacI* mutants recovered from Big Blue® transgenic animals. *Environ. Mol. Mutagen.*, **28**, 393–396.
23. Ushijima,T., Hosoya,Y., Suzuki,T., Sofini,T., Sugimura,T. and Nagao,M. (1995) A rapid method for determination of mutants in *lacI* gene using PCR-single strand conformation polymorphism analysis: demonstration of its high sensitivity. *Mutat. Res.*, **334**, 283–292.
24. Randerath,K., Haglund,D.H., Phillips,D.H. and Reddy,M.V. (1984) ^{32}P-post-labeling analysis of DNA adducts formed in livers of animals treated with safrol, estragole and other naturally-occurring alkenylbenzenes I. Adult female CD-1 mice. *Carcinogenesis*, **5**, 1613–1622.
25. Randerath,K., Reddy,M.V. and Gupta,R.C. (1981) ^{32}P-post-labeling test for DNA damage. *Proc. Natl Acad. Sci. USA*, **78**, 6126–6129.
26. Reddy,M.V. and Randerath,K. (1986) Nuclease P-1-mediated enhancement of sensitivity of ^{32}P-post-labeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543–1551.
27. Gupta,R.C., Reddy,M.V. and Randerath,K. (1982) ^{32}P-post-labeling analysis of non radioactive aromatic carcinogen–DNA adducts. *Carcinogenesis*, **3**, 1081–1092.
28. Reddy,M.V., Gupta,R.C., Randerath,E. and Randerath,K. (1984) ^{32}P-post-labeling test for covalent DNA binding of chemicals *in vivo*: Application to a variety of aromatic carcinogens and methylating agents. *Carcinogenesis*, **5**, 231–243.
29. Marmur,J. (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.*, **3**, 208–218.
30. Nagashima,M., Kasai,H., Yokota,J., Nagamachi,Y., Ichinose,T. and Sagai,M. (1995) Formation of an oxidative DNA damage, 8-hydroxydeoxyguanosine, in mouse lung DNA after intratracheal instillation of diesel exhaust particles and effects of high dietary fat and beta-cartene on this process. *Carcinogenesis*, **16**, 1441–1445.
31. Floyd,R.A., Watson,J.J., Wong,P.K., Altmiller,D.H. and Rickard,R.C. (1986) Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. *Free Radical Res. Commun.*, **1**, 163–172.
32. Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
33. Innis,M.A., Gelfand,G.H., Sininsky,J.J. and White,T.J. (1990) *PCR Protocols: a Guide to Methods and Applications*. Academic Press Inc, San Diego, CA.
34. Nebert,D.W. and Negishi,M. (1982) Multiple forms of cytochrome P-450 and the importance of molecular biology and evolution. *Biochem. Pharmacol.*, **31**, 2311–2317.
35. Krebs,O., Schafer,B., Wolff,T., Oestele,E., Deml,E., Sund,M. and Favor,J. (1998) The DNA damaging drug cyproterone acetate causes gene mutations and induces glutathione-*S*-transferase P in the liver of female Big Blue® F344 rats. *Carcinogenesis*, **19**, 241–245.
36. Murata-Kamiya,N., Kamiya,H., Kaji,H. and Kasai,H. (1997) Mutational specificity of glyoxal, a product of DNA oxidation, in the *lacI* gene of wild-type *Escherichia coli* W3110. *Mutat. Res.*, **377**, 255–262.
37. Ono,T., Negishi,K. and Hayatsu,H. (1995) Spectra of superoxide-induced mutations in the *lacI* gene of a wild-type and a *mut*M strain of *Escherichia coli* K-12. *Mutat. Res.*, **326**, 175–183.
38. Nikula,K.J., Snipes,M.B., Barr,E.B., Griffith,W.C., Henderson,R.F. and Mauderly,J.L. (1995) Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. *Fund. Appl. Toxicol.*, **25**, 80–94.
39. Gallagher,J., George,M., Kohan,M., Thompson,C., Shank,T. and Lewtas,J. (1993) Detection and comparison of DNA adducts after *in vitro* and *in vivo* diesel emission exposures. *Environ. Health Perspect.*, **99**, 225–228.
40. Hemminki,K., Soderling,J., Ericson,P., Norbeck,H.E. and Segerback,D. (1994) DNA adducts among personnel servicing and loading diesel vehicles. *Carcinogenesis*, **15**, 767–769.
41. Perera,F.P. (1988) The significance of DNA and protein adducts in human biomonitoring studies. *Mutat. Res.*, **205**, 255–269.
42. Harris,C.C. (1985) Future directions in the use of DNA adducts as internal dosimeters for monitoring human exposure to environmental mutagens and carcinogens. *Environ. Health Perspect.*, **62**, 185–191.
43. Sukumar,S., Natario,V., Martin-Zanca,D. and Barbacid,M. (1983) Induction of mammary carcinomas in rats by nitrosomethylurea involves malignant activation of H-*ras*-1 locus by single point mutations. *Nature*, **306**, 658–661.
44. Balman,A., Ramsden,M., Bowden,G.T. and Smith,J. (1984) Activation of the mouse cellular Harvey *ras* oncogene in chemically induced benign skin papillomas. *Nature*, **307**, 658–660.
45. Bailleul,B., Brown,K., Ramsden,M., Akhurst,R.J., Fee,F. and Balmain,A. (1989) Chemical induction of oncogene mutations and growth factor activity in mouse skin carcinogenesis. *Environ. Health Perspect.*, **81**, 23–27.
46. Harris,C.C. (1991) Chemical and physical carcinogenesis: Advances and perspectives for the 1990s. *Cancer Res. Suppl.*, **51**, 5023s–5044s.
47. Bond,J.A., Johnson,N.F., Snipes,M.B. and Mauderly,J.L. (1990) DNA adduct formation in rat alveolar type II cells: Cells potentially at risk for inhaled diesel exhaust. *Environ. Mol. Mutagen.*, **16**, 64–69.
48. Harris,C.C., Trump,B.F., Grafstrom,R. and Autrup,H. (1982) Differences in metabolism of chemical carcinogens in cultured human tissues and cells. *J. Cell. Biochem.*, **18**, 285–294.
49. Gotze,J.P., Lindeskog,P. and Tornquist,C.S.I. (1994) Effects of induction and age-dependent enzyme expression on lung bioavailability, metabolism and DNA binding of urban air particulate-absorbed benzo[*a*]pyrene, 2-nitrofluorene and 3-amino-1,4-dimethyl-5H-pyridol-(4,3)-indole. *Environ. Health Perspect.*, **102** (suppl.), 147–156.
50. Takahashi,Y. and Miura,T. (1989) Effects of nitrogen dioxide and ozone in combination on xenobiotic metabolizing activities of rat lungs. *Toxicology*, **56**, 253–262.
51. Yang,J.L., Maher,V.M. and McCormick,J.J. (1987) Kinds of mutations formed when a shuttle vector containing adducts of (+/–)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene replicates in human cells. *Proc. Natl Acad. Sci. USA*, **84**, 3787–3791.
52. Carothers,A.M. and Grunberger,D. (1990) DNA base changes in

benzo(*a*)pyrene diol epoxide-induced dihydrofolate reductase mutants of Chinese hamster ovary cells. *Carcinogenesis*, **11**, 189–192.
53. Bigger,C.A., Flickinger,D.J., St John,J., Harvey,R.G. and Dipple,A. (1991) Preferential mutagenesis at G:C base pairs by the anti 3,4-dihydrodiol 1,2-epoxide of 7-methylbenz[*a*]anthracene. *Mol. Carcinog.*, **4**, 176–179.
54. Bigger,C.A., Flickinger,D.J., Strandberg,J., Pataki,J., Harvey,R.G. and Dipple,A. (1990) Mutational specificity of the anti 1,2-dihydrodiol 3,4-epoxide of 5-methylchrysene. *Carcinogenesis*, **11**, 2263–2265.
55. Wei,S.J., Chang,R.L., Wong,C.Q., Bhachech,N., Cui,X.X., Hennig,E., Yagi,H., Sayer,J.M., Jerina,D.M., Preston,B.D. and Conney,A.H. (1991) Dose-dependent differences in the profile of mutations induced by an ultimate carcinogen from benzo[*a*]pyrene. *Proc. Natl Acad. Sci. USA*, **15**, 11227–11230.
56. Monroe,J.J., Kort,K.L., Miller,J.E., Marino,D.R. and Skopek,T.R. (1998) A comparative study of *in vivo* mutation assays: analysis of *hprt*, *lacI* and *cII/cI* as mutational targets for *N*-nitroso-*N*-methylurea and benzo[*a*]pyrene in Big Blue® mice. *Mutat. Res.*, **12**, 121–136.
57. Morita,M. (1993) Single-strand shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted G:C→T:A transversions on simian kidney cells. *Proc. Natl Acad. Sci. USA*, **90**, 1122–1126.
58. Kamiya,H. and Kasai,H. (1997) Mutations induced by 2-hydroxyadenine on a shuttle vector during leading and lagging strand syntheses in mammalian cells. *Biochemistry*, **36**, 11125–11130.
59. Smith,B.A., Manjanatha,M.G., Pogribny,I.P., Mittelstaedt,R.A., Chen,M.T., Fullerton,N.F., Beland,F.A. and Heflich,R.H. (1997) Analysis of mutations in the k-*ras* and *p53* genes of lung tumors and in the *hprt* gene of 6-thioguanine-resistant T-lymphocytes from rats treated with 1,6-dinitropyrene. *Mutat. Res.*, **379**, 61–68.
60. Mullin,A.H., Nataraj,D., Ren,J.J. and Mullin,D.A. (1998) Inhaled benzene increases the frequency and length of *lacI* deletions in lung tissues of mice. *Carcinogenesis*, **19**, 1723–1733.
61. Akiyama,N., Alexander,D., Aoki,Y. and Noda,M. (1996) Characterization of mutations induced by 300 and 320 nm UV radiation in a rat fibroblast cell line. *Mutat. Res.*, **372**, 119–131.
62. Bowden, D,H. (1983) Cell turnover in the lung. *Am. Rev. Respir. Dis.*, **128** (2 Pt 2), S46–S48.
63. Leslie,C.C., McCormick-Shannon,K., Cook,J.L. and Mason,R.J. (1985) Macrophages stimulate DNA synthesis in rat alveolar type II cells. *Am. Rev. Respir. Dis.*, **132**, 1246–1252.
64. White,H.J. and Garg,B.D. (1981) Early pulmonary response of the rat lung to inhalation of high concentration of diesel particles. *J. Appl. Toxicol.*, **1**, 104–110.
65. Schuetzle,D. (1983) Sampling of vehicle emissions for chemical analysis and biological testing. *Environ. Health Perspect.*, **47**, 65–80.

Received February 9, 1999; revised August 4, 1999; accepted November 26, 1999